PULLULANASE VARIANTS AND METHODS FOR PREPARING SUCH VARIANTS WITH PREDETERMINED PROPERTIES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a division of US Application No. 09/514,599 filed February 28, 2000 and claims, under 35 U.S.C. 119, priority of Danish application no. PA 2000 00045 filed January 12, 2000, the contents of which are fully incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates to variants of pullulanases and to methods for constructing such variants.

BACKGROUND OF THE INVENTION

Starches such as corn, potato, wheat, manioc and rice starch are used as the starting material in commercial large scale production of sugars, such as high fructose syrup, high maltose syrup, maltodextrins, amylose, G4-G6 oligosaccharides and other carbohydrate products such as fat replacers.

Degradation of starch

Starch usually consists of about 80% amylopectin and 20% amylose. Amylopectin is a branched polysaccharide in which linear chains Δ-1,4 D-glucose residues are joined by Δ-1,6 glucosidic linkages. Amylopectin is partially degraded by Δ-amylase, which hydrolyzes the 1,4-Δ-glucosidic linkages to produce branched and linear oligosaccharides. Prolonged degradation of amylopectin by Δ-amylase results in the formation of so-called Δ-limit dextrins which are not susceptible to further hydrolysis by the Δ-amylase. Branched oligosaccharides can be hydrolyzed into linear oligosaccharides by a debranching enzyme. The remaining branched oligosaccharides

can be depolymerized to D-glucose by glucoamylase, which hydrolyzes linear oligosaccharides into D-glucose.

Amylose is a linear polysaccharide built up of D-glucopyranose units linked together by α-1,4 glucosidic linkages. Amylose is degraded into shorter linear oligosaccharides by α-amylase, the linear oligosaccharides being depolymerized into D-glucose by glucoamylase.

In the case of converting starch into a sugar, the starch is depolymerized. The depolymerization process consists of a pretreatment step and two or three consecutive process steps, namely a liquefaction process, a saccharification process and, depending on the desired end product, optionally an isomerization process.

Pre-treatment of native starch

Native starch consists of microscopic granules which are insoluble in water at room temperature. When an aqueous starch slurry is heated, the granules swell and eventually burst, dispersing the starch molecules into the solution. During this "gelatinization" process there is a dramatic increase in viscosity. As the solids level is 30-40% in a typical industrial process, the starch has to be thinned or "liquefied" so that it can be handled. This reduction in viscosity is today mostly obtained by enzymatic degradation.

Liquefaction

During the liquefaction step, the long-chained starch is degraded into smaller branched and linear units (maltodextrins) by an α-amylase (*e.g.* Termamyl™, available from Novo Nordisk A/S, Denmark). The liquefaction process is typically carried out at about 105-110°C for about 5 to 10 minutes followed by about 1-2 hours at about 95°C. The pH generally lies between about 5.5 and 6.2. In order to ensure an optimal enzyme stability under these conditions, calcium is added, e.g. 1 mM of calcium (40 ppm free calcium ions). After this treatment the liquefied starch will have a "dextrose equivalent" (DE) of 10-15.

Saccharification

After the liquefaction process the maltodextrins are converted into dextrose by addition of a glucoamylase (e.g. AMG™, available from Novo Nordisk A/S) and a debranching enzyme, such as an isoamylase (see e.g. US Patent No. 4,335,208) or a pullulanase (*e.g.* Promozyme®, available from Novo Nordisk A/S; see US Patent No. 4,560,651). Before this step the pH is reduced to a value below 4.5, e.g. about 3.8, maintaining the high temperature (above 95°C) for a period of e.g. about 30 min. to inactivate the liquefying α-amylase to reduce the formation of short oligosaccharides called "panose precursors" which cannot be hydrolyzed properly by the debranching enzyme.

The temperature is then lowered to 60°C, glucoamylase and debranching enzyme are added, and the saccharification process proceeds for about 24-72 hours.

Normally, when denaturing the α-amylase after the liquefaction step, a small amount of the product comprises panose precursurs which cannot be degraded by pullulanases or AMG. If active amylase from the liquefaction step is present during saccharification (i.e. no denaturing), this level can be as high as 1-2% or even higher, which is highly undesirable as it lowers the saccharification yield significantly. For this reason, it is also preferred that the α-amylase is one which is capable of degrading the starch molecules into long, branched oligosaccharides (such as, e.g., the Fungamyl™-like α-amylases) rather than shorter branched oligosaccharides.

Isomerization

When the desired final sugar product is e.g. high fructose syrup, the dextrose syrup may be converted into fructose by enzymatic isomerization. After the saccharification process the pH is increased to a value in the range of 6-8, preferably about pH 7.5, and the calcium is removed by ion exchange. The dextrose syrup is then converted into high fructose syrup using, e.g., an immobilized glucose isomerase (such as Sweetzyme™, available from Novo Nordisk A/S).

Debranching enzymes

Debranching enzymes which can attack amylopectin are divided into two classes: isoamylases (E.C. 3.2.1.68) and pullulanases (E.C. 3.2.1.41), respectively. Isoamylase hydrolyses α-1,6-D-glucosidic branch linkages in amylopectin and β-limit dextrins and can be distinguished from pullulanases by the inability of isoamylase to attack pullulan, and by their limited action on α-limit dextrins.

When an acidic stabilized α-amylase is used for the purpose of maintaining the amylase activity during the entire saccharification process (no inactivation), the degradation specificity should be taken into consideration. It is desirable in this regard to maintain the α-amylase activity throughout the saccharification process, since this allows a reduction in the amyloglucidase addition, which is economically beneficial and reduces the AMG™ condensation product isomaltose, thereby increasing the DE (dextrose equivalent) yield.

It will be apparent from the above discussion that the known starch conversion processes are performed in a series of steps, due to the different requirements of the various enzymes in terms of e.g. temperature and pH. It would therefore be desirable to be able to engineer one or more of these enzymes, e.g. pullulanases, so that the overall process could be performed in a more economical and efficient manner. One possibility in this regard is to engineer the otherwise thermolabile pullulanases so as to render them more stable at higher temperatures.

BRIEF DISCLOSURE OF THE INVENTION

The inventors have modified the amino acid sequence of a pullulanase to obtain variants with improved properties, based on the three-dimensional structure of the pullulanase Promozyme® (available from Novo Nordisk A/S). The variants have altered physicochemical properties, e.g. an altered pH optimum, improved thermostability, increased specific activity or an altered cleavage pattern.

Accordingly, the object of the present invention is to provide a method for constructing pullulanases having altered properties, in particular to provide a method for constructing

pullulanases having improved thermostability, altered pH dependent activity and/or altered substrate specificity, such as increased isoamylase activity.

Thus, in its broadest aspect, the present invention relates to a method for constructing a variant of a parent pullulanase, wherein the variant has at least one altered property as compared to said parent pullulanase, which method comprises:

i) analyzing the structure of the pullulanase to identify, on the basis of an evaluation of structural considerations, at least one amino acid residue or at least one structural region of the pullulanase, which is of relevance for altering said property;

ii) constructing a variant of the pullulanase, which as compared to the parent pullulanase, has been modified in the amino acid residue or structural part identified in i) so as to alter said property; and

iii) testing the resulting pullulanase variant for said property.

The property which may be altered by the above methods of the present invention may be, e.g., thermostability, pH dependent activity, specific activity, or substrate specificity. Thus, the variant may have, e.g., increased thermostability, higher activity at a lower pH, an altered pH optimum, improved thermostability, or increased specific activity, such as increased isoamylase activity.

Although it has been described in the following that modification of the parent pullulanase in certain regions and/or positions is expected to confer a particular effect to the thus produced pullulanase variant (such as an improved thermostability or an increased isoamylase activity), it should be noted that modification of the parent pullulanase in any of such regions may also give rise to any other of the above-mentioned effects. For example, any of the regions and/or positions mentioned as being of particular interest with respect to, e.g., improved thermostability, may also give rise to, e.g., higher activity at a lower pH, an altered pH optimum, or increased specific activity, such as increased isoamylase activity.

Further aspects of the present invention relates to variants of a pullulanase, the DNA encoding such variants and methods of preparing the variants. Still further aspects of the present invention relates to the use of the variants for various industrial purposes, in particular for processes where sweeteners are made from starch. Other aspects of the present invention will be apparent from the below description as well as from the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the DNA plasmid pCA36 harboring the gene encoding the pullulanase from *Bacillus deramificans* (SEQ ID NO: 3).

DETAILED DISCLOSURE OF THE INVENTION

Pullulanases

As explained above, pullulanases are enzymes classified in EC 3.2.1.41 and such enzymes are characterized by their ability to hydrolyze the α-1,6-glycosidic bonds in, for example, amylopectin and pullulan.

A particularly interesting pullulanase is the pullulanase from *Bacillus acidopullulyticus* described in US 4,560,651 (hereinafter referred to as Promozyme®). Promozyme® has the amino acid sequence set forth in amino acids 1-921 of SEQ ID NO: 1. The three-dimensional structure of Promozyme® is described below.

Another interesting pullulanase is the pullulanase from *Bacillus deramificans* described in US 5,736,375. This enzyme has the amino acid sequence set forth in amino acid sequence 1-928 of SEQ ID NO: 3. Homology building of the tree-dimensional structure of the above-mentioned pullulanase is described below.

In general, a preferred pullulanase suitable for the purpose described herein should have one or more of the following properties:

i) A three-dimensional structure homologous to Promozyme®.

ii) An amino acid sequence which is at least 40% homologous to SEQ ID NO:1 or SEQ ID NO:3, preferably at least 50%, e.g. at least 60%, such as a least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% homologous to SEQ ID NO:1 or SEQ ID NO:3.

iii) A nucleic acid sequence which hybridizes to the nucleic acid sequence set forth in SEQ ID NO:1 or SEQ ID NO:3.

The structural homology referred to above in i) above is based on other sequence homologies, hydrophobic cluster analysis or by reverse threading (Huber, T ; Torda, AE, PROTEIN SCIENCE Vol. 7 , No. 1 pp. 142-149 (1998)) and which by any of these

methods is predicted to have the same tertiary structure as Promozyme, wherein the tertiary structure refers to the overall folding or the folding of Domains N1, N2, A, B, and C. Alternatively, a structural alignment between Promozyme and homologous sequences may be used to identify equivalent positions.

For example, the homology between various pullulanase with known amino acid sequence has been compiled in the below matrix:

	1	2	3	4	5	6	7	8	9	10
1. pula_kleae	100	86	59	51	52	53	52	52	55	50
2. pula_klepn		100	58	51	51	53	53	53	53	52
3. w81973			100	55	56	52	55	54	51	56
4. r56989				100	98	60	76	54	56	76
5. sp929mat					100	61	78	54	57	78
6. fervido_x						100	61	57	54	62
7. sp734							100	56	54	91
8. r71616								100	54	56
9. w09257									100	54
10. Promozyme®										100

1. Pula_kleae: Pullulanase from *Klebsiella aerogenes* (*J. Bacteriol.* (1987) **169**, 2301-2306).

2. Pula_klepn: Pullulanase from *Klebsiella pneumonia* (*Mol. Microbiol.* (1990) **4**, 73-85; *J. Bacteriol.* (1985) **164**, 639-645; *J. Bacteriol.* (1989) **171**, 3673-3679).

3. W81973: Pullulanase fragment from zea mays (WO 98/50562).

4. r56989: Mature pullulanase from *Bacillus deramificans* T 89.117D (EP 0 605 040).

5. sp929mat: Mature part of pullulanase from *Bacillus deramificans* (US 5,736,375).

6. fervido_x: Mature part of pullulanase from *Fervidobacterium pennavorans* Ven5 (*Appl. Environ. Microb.* (1997) **63**, 1088-1094).

7. sp734: Mature pullulanase from *Bacillus acidopullulyticus* (*FEMS Mic. Let.* (1994) **115**, 97-106.

8. r71616: Pullulanase from *Thermus sp.* (JP 07023783).

9. w09257: Pullulanase from *Bacillus sp.* KSM-AP 1378 (WO 96/35794).

The above homology calculations were determined by use of the GAP program from the UWGCG package using default values for GAP penalties, i.e. GAP creation penalty of 3.0 and GAP extension penalty of 0.1 (Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711).

A sequence alignment between Promozyme® (SEQ ID NO: 1), the pullulanase from *Bacillus deramificans* (SEQ ID NO: 3) and the pullulanase from *Bacillus acidopullulyticus* (SEQ ID NO: 5) described in *FEMS Mic. Let.* (1994) **115**, 97-106, is shown in Appendix 2.

Three-dimensional structure of pullulanase

Promozyme® was used to elucidate the three-dimensional structure forming the basis for the present invention.

The structure of Promozyme® was solved in accordance with the principle for x-ray crystallographic methods, for example, as given in X-Ray Structure Determination, Stout, G.K. and Jensen, L.H., John Wiley & Sons, Inc. NY, 1989.

The structural coordinates for the solved crystal structure of Promozyme® using the isomorphous replacement method are given in standard PDB format (Protein Data Bank, Brookhaven National Laboratory, Brookhaven, CT) as set forth in Appendix 1. It is to be understood that Appendix 1 forms part of the present application. In the context of Appendix 1, the following abbreviations are used: WAT refers to water or to calcium. Amino acid residues are given in their standard three letter code.

The structure of said Pullulanase is made up of five globular domains, ordered N1, N2, A, B, and C. The domains can be defined as being residues 1-310 for domain N1, 311-

420 for Domain N2, residues 421-556 and 596-835 for domain A, residues 557-595 for Domain B, residues 596-922 for Domain C, wherein the numbering refers to the amino acid sequence in SEQ ID NO: 1. Features of Domains N1, A, B and C of particular interest are described below.

Domain N1

Domain N1 contains in this particular pullulanase an extra loop of 100 residues compared to the pullulanase from *Bacillus acidopullulyticus* having the amino acid sequence shown in SEQ ID NO: 5. The loop is also present in the pullulanase from *Bacillus deramificans* having the amino acid sequence shown in SEQ ID NO: 3.

Part of the N2 domain is homologeous to the N1 domain of Pseudomonase amyloderamosa isoamylase (1bf2.pdb from Brookhaven database).

Domain A

Domain A is the largest domain and contains the active site which comprises a cluster of three amino acid residues, D622, D736 and E651, spatially arranged at the bottom of a cleft in the surface of the enzyme. The structure of Domain A shows an overall fold in common with the α-amylases for which the structure is known, viz. the (beta/alpha) 8 barrel with eight central beta strands (numbered 1-8) and eight flanking a-helices. The β-barrel is defined by McGregor, *J. Prot. Chem.* 7:399, 1988. The C-terminal end of the beta strand 1 is connected to helix 1 by a loop denoted loop 1 and an identical pattern is found for the other loops, although the loops show some variation in size and some can be quite extensive.

The eight central beta-strands in the (beta/alpha) 8 barrel superimpose reasonably well with the known structures of family 13 (Henrissat B. *Biochem. J.* (1991) **280**, 309-316 and Henrissat B. and Bairoch A. *Biochem. J.* (1993) **293**, 781-788). This part of the structure, including the close surroundings of the active site located at the C-terminal end of the beta-strands, shows a high degree of homology with isoamylases.

In contrast, the loops connecting the beta-strands and alpha helices display a high degree of variation from the known structures of family 13 enzymes. These loops constitute the structural context of the active site, and the majority of the contacts to the substrate is found among residues located in these loops. Distinguishing characteristics such as substrate specificity, substrate binding, pH activity profile, substrate cleavage pattern, and the like, are determined by specific amino acids and the positions they occupy in these loops.

Domain B

Domain B, also referred to as loop 3 of the (beta/alpha) 8 barrel, in comprises amino acid residues 557-595 of the amino acid sequence shown in SEQ ID NO: 1. The most striking difference to other family 13 enzymes being the short amino acid sequence. This short sequence loop are of the same size as the isoamylase loop 3 and spatially positioned close to the active site residues and in close contact to the substrate.

Domain C

Domain C in Promozyme comprises amino acid residues 596-922 of the amino acid sequence shown in SEQ ID NO: 1. Domain C is composed entirely of β-strands which form a single 8-stranded sheet structure that folds back on itself, and thus may be described as a β-sandwich structure. One part of the β-sheet forms the interface to Domain A.

Substrate Binding Site

Parts of the loop discussed above in the context of domains A, B and N2 are of particular interest for substrate interaction and active site reactivity. In particular, in domain A, residues 439-443 in loop 1, residues 490-514 in loop 2, residues 621-628 in loop 4, residues 652-668 in loop 5, residues 679-694 in loop 6, residues 733-740 in loop 7 and residues 787-796 in loop 8; in domain B, residues 553-564 and 581-592 in loop 3; in domain N2, residues 400-404, wherein residue positions correspond to the amino acids in the amino acid sequence in SEQ ID NO: 1.

Homology building of *Bacillus deramificans* pullulanase or other pullulanases.

The structure of the *Bacillus deramificans* pullulanase (SEQ ID NO:3) was model built on the structure disclosed in Appendix 1 herein. The structure of other pullulanases may be built analogously.

A model structure of a pullulanase can be built using the Homology program or a comparable program, e.g., Modeller (both from Molecular Simulations, Inc., San Diego, CA). The principle is to align the sequence of the pullulanase with the known structure with that of the pullulanase for which a model structure is to be constructed. The structurally conserved regions can then be built on the basis of consensus sequences. In areas lacking homology, loop structures can be inserted, or sequences can be deleted with subsequent bonding of the necessary residues using, e.g., the program Homology. Subsequent relaxing and optimization of the structure should be done using either Homology or another molecular simulation program, e.g., CHARMm from Molecular Simulations.

Methods for designing novel pullulanase variants

In a first aspect, the present invention relates to a method for producing a variant of a parent pullulanase, wherein the variant has at least one altered property as compared to the parent pullulanase, the method comprising:

i) modeling the parent pullulanase on the three-dimensional structure of SEQ ID NO: 1 depicted in Appendix 1 to produce a three-dimensional structure of the parent pullulanase;

ii) identifying in the three-dimensional structure obtained in step (i) at least one structural part of the parent pullulanase, wherein an alteration in the structural part is predicted to result in an altered property;

iii) modifying the nucleic acid sequence encoding the parent pullulanase to produce a nucleic acid sequence encoding a deletion, insertion, or substitution of one or more amino acids at a position corresponding to the structural part; and

iv) expressing the modified nucleic acid sequence in a host cell to produce the variant pullulanase.

The structural part which is identified in step ii) of the method of the invention may be composed of one amino acid residue. Normally, however, the structural part comprises more than one amino acid residue, typically constituting one of the above-mentioned parts of the pullulanase structure such as one of the N1, N2, A, B, or C domains, an interface between any of these domains, a calcium binding site, a loop structure, the substrate binding site, or the like.

The structural or functional considerations may involve an analysis of the relevant structure or structural part and its contemplated impact on the function of the enzyme. For example, an analysis of the functional differences between pullulanases and the various isoamylases may be used for assigning certain properties of Promozyme® or homologeous model builded structure to certain parts of the Promozyme® or homologeous model builded structure or to contemplate such relationship. For instance, differences in the pattern or structure of loops surrounding the active site may result in differences in access to the active site of the substrate and thus differences in substrate specificity and/or cleavage pattern.

Furthermore, parts of a pullulanase involved in substrate binding, and thus, for example, substrate specificity and/or cleavage, thermostability, and the like, have been identified (*vide infra*).

The modification of an amino acid residue or structural region is typically accomplished by suitable modifications of a nucleic acid sequence encoding the parent enzyme in question. The modification may be substitution, deletion or insertion of an amino acid residue or a structural part.

The property to be modified may be stability (e.g. thermostability), pH dependent activity, substrate specificity, such as decreased condensation reactions, isoamylase like activity etc. Thus, the altered property may be an altered specific activity at a given

pH and/or altered substrate specificity, such as an altered pattern of substrate cleavage or an altered pattern of substrate inhibition.

In step ii) of the method according to the invention the part of the structure to be identified is preferably one which in the folded enzyme is believed to be in contact with the substrate (cf. the disclosure above in the section entitled "Substrate Binding Site") or involved in substrate specificity and/or cleavage pattern, and/or one which is contributing to the pH or temperature profile of the enzyme, or is otherwise responsible for the properties of the pullulanase.

Described in the following are specific types of variants which have been designed by use of the method of the invention.

The variants of the invention may comprise additional modifications in addition to the modifications described herein. The variants preferably have an amino acid sequence having more than 40% homology with SEQ ID NO: 1, SEQ ID NO: 3, or SEQ ID NO: 5, preferably more than 50%, e.g. more than 60%, such as more than 70%, more than 75%, more than 80%, more than 85%, more than 90%, more than 91%, more than 92%, more than 93%, more than 94%, more than 95%, more than 96%, more than 97%, more than 98% or more than 99% homology with the amino acid sequences shown in SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 5.

In the present context the term "homologous to" or "homology" (also sometimes referred to as "similarity") is used in it conventional meaning and the "homology" between two amino acid sequences may be determined by use of any conventional algorithm, preferably by use of the GAP program from the UWGCG package using default values for GAP penalties, i.e. GAP creation penalty of 3.0 and GAP extension penalty of 0.1 (Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711). The method is also described in S.B. Needleman and C.D. Wunsch, *Journal of Molecular Biology*, **48**, 443-445 (1970).

As mentioned above, the property to be modified may be stability (e.g. thermostability), pH dependent activity, substrate specificity, such as increased isoamylase activity, or specific activity. Thus, the altered property may be an altered specific activity at a given pH and/or an altered substrate specificity, such as an altered pattern of substrate cleavage or an altered pattern of substrate inhibition.

In a particular interesting embodiment of the invention the property to be modified is the thermostability of the enzyme.

In the present context, the term "thermostable" (or "thermostability") refers in general to the fact that the pullulanase variants according to the invention have an improved thermostability compared to the relevant parent pullulanase. The degree of improvement in thermostability can vary according to factors such as the thermostability of the parent pullulanase and the intended use of the pullulanase variant, i.e. whether it is primarily intended to be used for liquefaction or for saccharification or both. It will be apparent from the discussion below that for saccharification, the enzyme variant should maintain a substantial degree of enzyme activity during the saccharification step at a temperature of at least about 63°C, preferably at least about 70°C, while an enzyme variant designed for use in the liquefaction step should be able to maintain a substantial degree of enzyme activity at a temperature of at least about 95°C.

The improved thermostability of enzyme variants according to the invention can in particular be defined according to one or more of the following criteria:

In one embodiment, the pullulanase variant of the invention has an improved thermostability (and/or the method of the invention provides a pullulanase with an improved thermostabilty) as defined by differential scanning calorimetry (DSC) using the method described herein.

In another embodiment, the pullulanase variant of the invention has an improved thermostability (and/or the method of the invention provides a pullulanase with an improved thermostabilty) as defined by an increased half-time ($T_{1/2}$) of at least about 5%, preferably at least about 10%, more preferably at least about 15%, more preferably

at least about 25%, most preferably at least about 50%, such as at least about 100%, in the "$T_{1/2}$ assay for liquefaction" described herein, using a pH of 5.0 and a temperature of 95°C. Pullulanase variants according to this definition are suitable for use in the liquefaction step of the starch conversion process.

Alternatively or additionally, a pullulanase variant suitable for use in liquefaction can be defined as having an improved thermostability as defined by an increased residual enzyme activity of at least about 5%, preferably at least about 10%, more preferably at least about 15%, more preferably at least about 25%, most preferably at least about 50%, such as at least about 100%, in the "assay for residual activity after liquefaction" described herein, using a pH of 5.0 and a temperature of 95°C.

In a further embodiment, the enzyme variant of the invention has an improved thermostability (and/or the method of the invention provides a pullulanase with an improved thermostabilty) as defined by an increased half-time ($T_{1/2}$) of at least about 5%, preferably at least about 10%, more preferably at least about 15%, more preferably at least about 25%, most preferably at least about 50%, such as at least about 100%, in the "$T_{1/2}$ assay for saccharification" described herein, using a pH of 4.5 and a temperature of 70°C. Such variants are suitable for use in the saccharification step of the starch conversion process.

Alternatively or additionally, a pullulanase variant suitable for saccharification can be defined as having an improved thermostability as defined by an increased residual enzyme activity of at least about 5%, preferably at least about 10%, more preferably at least about 15%, more preferably at least about 25%, most preferably at least about 50%, such as at least about 100%, in the "assay for residual activity after saccharification" described herein, using a pH of 4.5 and a temperature of 63°C. Preferably, this improved thermostability is also observed when assayed at a temperature of 70°C.

The term "substantially active" as used herein for a given pullulanase variant and a given set of conditions of temperature, pH and time means that the relative enzymatic

activity of the enzyme variant is at least about 25%, preferably at least about 50%, in particular at least about 60%, especially at least about 70%, such as at least about 90% or 95%, e.g. at least about 99% compared to the relative activity of the parent enzyme tested under the same set of conditions.

One advantage of the thermostable pullulanase of the invention is that they make it possible to perform liquefaction and debranching simultaneously before the saccharification step. This has not previously been possible, since the known pullulanases with acceptable specific activity are thermolabile and are inactivated at temperatures above 60°C. (Some thermostable pullulanases from *Pyrococcus* are known, but these have an extremely low specific activity at higher temperatures and are thus unsuitable for purposes of the present invention). By debranching, using the thermostable pullulanases of the invention, during liquefaction together with the action of an α-amylase, the formation of panose precursors is reduced, thereby reducing the panose content in the final product and increasing the overall saccharification yield. It is also possible in this manner to extend the liquefaction process time without risking formation of large amount of panose precursors. By prolonging the liquefaction step, the DE yield is increased from 10-15 to e.g. 15-20, reducing the need for glucoamylase. This reduced glucoamylase requirement is in turn advantageous as the formation of undesired isomaltose is reduced, thereby resulting in an increased glucose yield. In addition, the reduced glucoamylase addition enables the saccharification step to be carried out at a higher substrate concentration (higher DS, dry substances, concentration) than the normal approx. 30-35% used according to the prior art. This allows reduced evaporation costs downstream, e.g. in a high fructose corn syrup process, and the saccharification reaction time can also be reduced, thereby increasing production capacity. A further advantage is that α-amylase used in the liquefaction process does not need to be inactivated/denatured in this case.

Furthermore, it is also possible to use the thermostable pullulanases of the invention during saccharification, which is advantageous for several reasons. In the conventional starch saccharification process, the process temperature is not more than 60°C due to the fact that neither the saccharification enzyme pullulanase nor AMG™ are sufficiently

thermostable to allow the use of a higher temperature. This is a disadvantage, however, as it would be very desirable to run the process at a temperature of above about 60°C, in particular above 63°C, e.g. about 70°C, to reduce microbial growth during the relatively long saccharification step. Furthermore, a higher process temperature normally gives a higher activity per mg of enzyme (higher specific activity), thereby making it possible to reduce the weight amount of enzyme used and/or obtain a higher total enzymatic activity. A higher temperature can also result in a higher dry matter content after saccharification, which would be beneficial in terms of reducing evaporation costs.

In another interesting embodiment of the invention the property to be modified is the substrate specificity of the pullulanase, in particular to modify the substrate specificity of the pullulanase in such a way the variant pullulanase becomes more "isoamylase-like" in the sense of having an increased activity towards high molecular weight branched starchy material such as glycogen and amylopectin. Methods for determining the substrate specificity of pullulanases are discussed in the following section entitled "Methods for determing stability, activity and specificity".

Thus, when used herein, the term "increased isoamylase activity" refers in general to the fact that the pullulanase variants according to the invention exhibits a higher activity towards high molecular weight branched starchy material, such as glycogen and amylopectin as compared to the parent pullulanase.

The increased isoamylase activity of the pullulanase variants according to the invention can in particular be defined according to the below criteria:

In one embodiment the pullulanase variant according to the invention has an increased isoamylase activity as defined by an increase of at least 5%, preferably of at least 10%, more preferably of at least 15%, more preferably of at least 25%, most preferably of at least 50%, in particular of at least 75%, such as of at least 100% in the number of reducing ends formed in the "assay for isoamylase-like activity" described herein, using 50 mM sodium acetate, a pH of 4.5, 5.0 or 5.5, a temperature of 60°C and when incubated with a 10 w/v rabbit liver glycogen solution for a period of 10 min.

In the present context the term "pullulanase activity" is intended to mean that the pullulanase variant in question is capable of degrading pullulan when tested as described in the Examples (see the section entitled "Determination of pullulanase activity).

Methods for determining stability, activity and specificity

Thermostability

Thermostability of pullulanases can be detected by measuring the residual activity by incubating the enzyme under accelerated stress conditions, which comprise: pH 4.5 in a 50 mM sodium acetate buffer without a stabilizing dextrin matrix (such as the approximately 35% dry matter which is normally present during saccharification). The stability can be determined at isotherms of e.g. 63°C, 70°C, 80°C, 90°C and 95°C, measuring the residual activity of samples taken from a water bath at regular intervals (e.g. every 5 or 10 min.) during a time period of 1 hour. For determining stability for the purpose of liquefaction, a pH of 5.0, a temperature of 95°C and a total assay time of 30 to 120 minutes are used ("assay for residual activity after liquefaction"). For determining stability for the purpose of saccharification, a pH of 4.5, a temperature of 63°C or 70°C and a total assay time of 30 minutes are used ("assay for residual activity after saccharification").

Alternatively, the thermostability may be expressed as a "half-time" ($T_{1/2}$), which is defined as the time, under a given set of conditions, at which the activity of the enzyme being assayed is reduced to 50% of the initial activity at the beginning of the assay. In this case, the "$T_{1/2}$ assay for liquefaction" uses a pH of 5.0 and a temperature of 95°C, while the "$T_{1/2}$ assay for saccharification" uses a pH of 4.5 and a temperature of 70°C. The assay is otherwise performed as described above for the respective assays for residual activity.

Activity: Somogyi-Nelson method for determination of reducing sugars

The activity of pullulanases can be measured using the Somogyi-Nelson method for the determination of reducing sugars (*J. Biol. Chem.* **153**, 375 (1944)). This method is based on the principle that sugar reduces cupric ions to cuprous oxide, which reacts with an arsenate molybdate reagent to produce a blue colour that is measured spectrophotometrically. The solution to be measured must contain 50-600 mg of glucose per liter. The procedure for the Somogyi-Nelson method is as follows:

Sample value: Pipet 1 ml of sugar solution into a test tube. Add 1 ml of copper reagent. Stopper the test tube with a glass bead. Place the test tube in a boiling water bath for 20 minutes. Cool the test tube. Add 1 ml of Nelson's color reagent. Shake the test tube without inverting it. Add 10 ml of de-ionized water. Invert the test tube and shake vigorously. Measure the absorbance at 520 nm, inverting the test tube once immediately prior to transfer of the liquid to the cuvette.

Blank value: Same procedure as for the sample value, but with water instead of sugar solution.

Standard value: Same procedure as for the sample value.

Calculations: In the region 0-2 the absorbance is proportional to the amount of sugar.

$$\text{mg sugar/l} = \frac{100\ (\text{sample} - \text{blank})}{(\text{standard} - \text{blank})}$$

$$\%\ \text{glucose} = \frac{(\text{sample} - \text{blank})}{100 \times (\text{standard} - \text{blank})}$$

Reagents:

1. Somogyi's copper reagent

35.1 g $Na_2HPO_4.2H_2O$ and 40.0 g potassium sodium tartrate ($KNaC_4H_4O_2.4H_2O$) are dissolved in 700 ml of de-ionized water. 100 ml of 1N sodium hydroxide and 80 ml of 10% cupric sulphate ($CuSO_4.5H_2O$) are added. 180 g of anhydrous sodium sulphate are dissolved in the mixture, and the volume is brought to 1 liter with de-ionized water.

2. Nelson's color reagent

50 g of ammonium molybdate are dissolved in 900 ml of de-ionized water. Then 42 ml of concentrated sulphuric acid are added, followed by 6 g of disodium hydrogen arsenate heptahydrate dissolved in 50 ml of deionized water, and the volume is brought to 1 litre with deionized water. The solution is allowed to stand for 24-48 hours at 37°C before use and is stored in the dark in a brown glass bottle with a glass stopper.

3. Standard

100 mg of glucose (anhydrous) are dissolved in 1 liter of de-ionized water.

Alternatively, the release of reducing sugars can be measured using a 96 well plate set-up modified after Fox, J.D. & Robyt, J.F. (1991) Anal. Biochem. 195, 93-96. Assay conditions are (in brief): 1 ml substrate (e.g. 1% solution) in 50 mM citric acid pH 5 is preincubated at 60 °C. A zero timepoint is taken 150 µl sample and transferred to a microtiter plate well containing 150 µl solution A + B for reducing sugar determination. The enzymatic reaction is initiated by addition of 100 µl enzyme and time points are taken at T = 1, 2, 3, 4, and 5 min.

After completion of the assay, the plate is developed by incubation at 85 °C for 70 minutes and the plate is read at 540 nm.

Reagents for determination of reducing value: Solution A) and solution B (62 mg copper sulfate pentahydrate and 63 mg L-serine in 50 ml water).

Pullulanase specificity

Methods for the determination and characterization of the profile of action and specificity of pullulanases for various substrates (e.g. amylopectin, glycogen and pullulan) are described by Kainuma et al. in *Carbohydrate Research,* **61** 345-357 (1978). Using these methods, the relative activity of a pullulanase can be determined, and the relative activity of a pullulanase variant according to the invention compared to the relative activity of the parent pullulanase can be assessed, for example to determine whether a pullulanase variant has the desired increased specificity toward high molecular weight saccharides, such as amylopectin, compared to the parent pullulanase.

In order to determine whether the pulluanase variant possesses an increased isoamylase activity as compared to the parent pullulanse the following test may be performed ("assay for isoamylase-like activity"):

1000 mg rabbit liver glycogen is dissolved in 40 ml water to which 0.2% NaOH has been added. 800 mg $NaBH_4$ is added carefully under stirring. The solution is stirred for 48 hours at 25°C after which the reaction is stopped by addition of Amberlite IR-118H (a cation exchanger which removes the boron ions and hence stops the reaction). The solution is filtered to remove the matrix and evaporated to give 10 ml. The solution is then dialyzed extensively against de-ionized water to remove residual boron ions. The parent pullulanase and the pulluanase variant are assayed according to the method of Somogyi-Nelson, using 50 mM sodium acetate, pH values of 4.5, 5.0 or 5.5 and a temperature of 60°C, with a reaction time of 10 minutes. Glucose is used as a standard, a standard curve being made from solutions containing of 0-200 mg glucose/liter.

Clearly, the higher the number of reducing ends formed during the incubation period, the higher "isoamylase activity". The increase in the pullulanase variant's isoamylase activity is expressed as a percentage value based on the original "isoamylase activity" of the parent pullulanase.

Pullulanase variants with altered stability

A variant with improved stability (typically increased thermostability) may be obtained by substitution with proline, substitution of histidine with another amino acid, introduction of a disulfide bond, removal of a deamidation site, altering a hydrogen bond contact, filling in an internal structural cavity with one or more amino acids with bulkier side groups, introduction of interdomain interactions, altering charge distribution, helix capping, or introduction of a salt bridge.

Increased mobility regions:

The following regions have an increased mobility in the crystal structure of Promozyme®, and it is presently believed that these regions can be responsible for stability or activity of the enzyme. Improvements of the enzyme can be obtained by mutation in the below regions and positions. Introducing e.g. larger residues or residues having more atoms in the side chain could increase the stability, or e.g. introduction of residues having fewer atoms in the side chain could be important for the mobility and thus the activity profile of the enzyme. The regions can be found by analysing the B-factors taken from the pdb file, and/or from molecular dynamics calculations of the isotropic fluctuations. These can be obtained by using the program CHARMm from MSI (Molecular simulations inc.).

Thus, in order to stabilize mobile regions in the structure, a preferred variant of a parent pullulanase comprises a modification, e.g. a substitution, of an amino acid residue corresponding to one or more of the following residues of the amino acid sequence set forth in SEQ ID NO: 1:

408-429 (i.e. 408, 409, 410, 411, 412, 413, 414, 415, 416, 417, 418, 419, 420, 421, 422, 423, 424, 425, 426, 427, 428 and 429),

300-314 (i.e. 300, 301, 302, 303, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313 and 314),

157-165 (i.e. 157, 158, 159, 160, 161, 162, 163, 164 and 165),

95-113 (i.e. 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112 and 113),

130-140 (i.e. 130, 131, 132, 133, 134, 135, 136, 137, 138, 139 and 140),

232-238 (i.e. 232, 233, 234, 235,236, 237 and 238),

266-272 (i.e. 266, 267, 268, 269, 270, 271 and 272),

302-308 (i.e. 302, 303, 304, 305, 306, 307 and 308),

418-428 (i.e. 418, 419, 420, 421, 422, 423, 424, 425, 426, 427 and 428),

500-507 (i.e. 500, 501, 502, 503, 504, 505, 506 and 507),

659-665 (i.e. 659, 660, 661, 662, 663, 664 and 665) and

751-755 (i.e. 751, 752, 753, 754 and 755).

Similar modifications, e.g. substitutions, may be introduced in equivalent positions of other pullulanases. Variants of particular interest have a combination of one or more of the above with any of the other modifications disclosed herein.

For example, other preferred modifications, e.g. substitutions, which are believed to stabilized mobile regions in the structure of the pullulanase from *Bacillus deramificans*, correspond to one or more of the following residues of the amino acid sequence set forth in SEQ ID NO: 3:

406-427 (i.e. 406, 407, 408, 409, 410, 411, 412, 413, 414, 415, 416, 417, 418, 419, 420, 421, 422, 423, 424, 425, 426 and 427),

298-312 (i.e. 298, 299, 300, 301, 302, 303, 304, 305, 306, 307, 308, 309, 310, 311 and 312),

153-161 (i.e. 153, 154, 155, 156, 157, 158, 159, 160 and 161),

91-109 (i.e. 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108 and 109),

126-136 (i.e. 126, 127, 128, 129, 130, 131, 132, 133, 134, 135 and 136),

230-236 (i.e. 230, 231, 232, 233, 234, 235 and 236),

264-270 (i.e. 264, 265, 266, 267, 268, 269 and 270),

300-306 (i.e. 300, 301, 302, 303, 304, 305 and 306),

416-426 (i.e. 416, 417, 418, 419, 420, 421, 422, 423, 424, 425 and 426),

498-505 (498, 499, 500, 501, 502, 503, 504 and 505),

656-662 (i.e. 656, 657, 658, 659, 660, 661 and 662) and

749-753 (i.e. 749, 750, 751, 752 and 753).

Furthermore, it is envisaged from the structure that deletion of certain amino acid residues will confer increased stability, such as increased thermostability, to the thus produced variant. Variants, which are believed to be of particular importance, comprises a deletion of amino acid residues corresponding to the following residues of the amino acid sequence set forth in SEQ ID NO: 1:

Deletion of the peptide fragment 158-275, such as a deletion starting from position 158, 159, 160 or 161 and ending at position 270, 271, 272, 273, 274 or 275, i.e. the longest deletion will be deletion of the peptide fragment 158-275 and the shortest deletion will be deletion of the peptide fragment 161-270.

Other deletions which are expected to confer increased stability, such as increased thermostability, to the pullulanase variant comprises a deletion of amino acid residues corresponding to the following residues of the amino acid sequence set forth in SEQ ID NO: 1:

Deletion of the peptide fragment 1-315, such as deletion of the peptide fragment 1-314, 1-313, 1-312, 1-311, 1-310, 1-309, 1-308, 1-307, 1-306, 1-305, or 1-304.

Furthermore, the following deletions are expected to confer increased stability, such as increased thermostability, to the pullulanase variant comprises a deletion of amino acid residues corresponding to the following residues of the amino acid sequence set forth in SEQ ID NO: 1:

Deletion of the peptide fragment 1-115, such as deletion of the peptide fragment 1-114, 1-113, 1-112, 1-111, 1-110, 1-109, 1-108, 1-107, 1-106 or 1-105.

Similar deletions may be introduced in equivalent positions of other pullulanases. Variants of particular interest have a combination of one or more of the above with any of the other modifications disclosed herein.

For example, it is envisaged that deletion of the below amino acid residues will confer increased stability, such as increased thermostability, to the thus produced variant of the pullulanase from *Bacillus deramificans* (SEQ ID NO: 3):

Deletion of the peptide fragment 154-273, such as a deletion starting from position 154, 155, 156 or 157 and ending at position 268, 269, 270, 271, 272 or 273, i.e. the longest deletion will be deletion of the peptide fragment 154-273 and the shortest deletion will be deletion of the peptide fragment 157-268.

Other deletions which are expected to confer increased stability, such as increased thermostability, to the pullulanase variant comprises a deletion of amino acid residues corresponding to the following residues of the amino acid sequence set forth in SEQ ID NO: 3:

Deletion of the peptide fragment 1-313, such as deletion of the peptide fragment 1-312, 1-311, 1-310, 1-309, 1-308, 1-307, 1-306, 1-305, 1-304, or 1-303.

Furthermore, the following deletions are expected to confer increased stability, such as increased thermostability, to the pullulanase variant comprises a deletion of amino acid residues corresponding to the following residues of the amino acid sequence set forth in SEQ ID NO: 3:

Deletion of the peptide fragment 1-111, such as deletion of the peptide fragment 1-111, 1-110, 1-109, 1-108, 1-107, 1-106, 1-105, 1-104, 1-103, 1-102 or 1-101.

Cavities and crevices

The structure of the pullulanase contains a number of unique internal cavities, which may contain water, and a number of crevices. In order to increase the stability, preferably the thermostability, of the pullulanase it may be desirable to reduce the number or size of cavities and crevices, e.g., by introducing one or more hydrophobic contacts, preferably achieved by introducing amino acids with bulkier side chains in the vicinity or surroundings of the cavity or crevice. For instance, the amino acid residues to be modified are those which are involved in the formation of a cavity or crevice.

In order to determine which amino acid residues of a given enzyme are involved in the formation of cavities or crevices the Conolly program is normally used (B. Lee and F.M. Richards, *J. Mol. Biol.* **55**, 379-400 (1971)). The program uses a probe with a certain radius to search the external and internal surface of the protein. The smallest crevice observable in this way has the probe radius.

To analyze the solved structure of Promozyme®, a modified version of the Connolly program included in the program of INSIGHT was used. In the first step, the water molecules and the ions were removed by unmerging these atoms from the solved structure. By using the command MOLECULE SURFACE SOLVENT the solvent accessible surface area was calculated for all atoms and residues using a probe radius of 1.4 Å, and displayed graphically together with the model of the solved structure. The internal cavities are then seen as dot surfaces with no connections to the external surface.

Suggestions for specific modifications to fill out the cavities are given below. By using the homology built structures and/or comparisons based on sequence alignment, mutations for homologous structures of pullulanases can be made.

Accordingly, in a further aspect the present invention relates to a method for constructing a variant of a parent pullulanase, the method comprising:

a) identifying an internal cavity or crevice in the three-dimensional structure of the parent pullulanase;

b) substituting at least one amino acid residue involved in the formation of a cavity or crevice with another amino acid residue which increases the hydrophobic interaction and/or fills out or reduces the size of the cavity or crevice;

c) optionally repeating steps a) and b) recursively;

d) optionally, making alterations each of which is an insertion, a deletion or a substitution of an amino acid residue at one or more positions other than b);

e) preparing the variant resulting from steps a) - d);

f) testing the stability and/or the temperature dependent activity profile of the variant; and

g) optionally repeating steps a) - f) recursively; and

h) selecting a variant having increased stability and/or an altered temperature dependent activity profile as compared to the parent pullulanase.

In a preferred embodiment of the invention the variant pullanase provided by the above method have increased thermostability as compared to the parent pullulanase. The thermostability of a given variant may be assessed as described in the above section entitled "Methods for determining stability, activity and specificity".

It will be understood that the cavity or crevice is identified by the amino acid residues surrounding said cavity or crevice, and that modification of said amino acid residues are of importance for filling or reducing the size of the cavity or crevice. Preferably, the modification is a substitution with a bulkier amino acid residue, i.e. one with a greater side chain volume or with an increased number of atoms in the side chain. For example, all the amino acids are bulkier than Gly, whereas Tyr and Trp are bulkier than Phe. The particular amino acid residues referred to below are those which in a crystal structure have been found to flank the cavity or crevice in question.

In a preferred embodiment, the variant of a pullulanase, in order to fill, either completely or partly, cavities or crevices located internally or externally in the structure, comprises a modification, e.g. a substitution, of an amino acid residue corresponding to one or more of the following residues of the amino acid sequence set forth in SEQ ID NO: 1:

406, 394, 568, 573 576, 563, 557, 396, 392, 515, 583, 442, 792, 767, 732, 760, 783, 740, 688, 478, 534, 550, 627, 314.

In a more preferred embodiment, the variant of a pullulanse comprises one or more substitutions corresponding to the following substitutions in the amino acid sequence set forth in SEQ ID NO: 1:

G406A, P394F/W/I/L, I568L/F, Y573W, T576N/L/I, S563T, T557N, A396V/L/I, V392, N515M/L/I, V583I/F/L, D442Q, S792Y/F, V767Q/E/L/I, V732I/L, D760Q/E/F/Y, L783F/Y, L740Q, D688Y/F/E/Q/R/K, L478Q/R, L534F/Y/I, M550F/Y/I/L, L627F/Y/I, L314I.

Similar modifications, e.g. substitutions, may be introduced in equivalent positions of other pullulanases. Variants of particular interest have a combination of one or more of the above with any of the other modifications disclosed herein.

For example, the variant of a pullulanase may also comprise one or more substitutions corresponding to the following substitutions in the amino acid sequence set forth in SEQ ID NO: 3:

566, 485, 487, 437, 775, 779, 551, 428, 492, 495, 392, 621, 437+503, 674+664 and 823.

In a more preferred embodiment, the variant of a pullulanse comprises one or more substitutions corresponding to the following substitutions in the amino acid sequence set forth in SEQ ID NO: 3:

I566A, Q485H, M487L, D437H, Q775H, E779D, V551I, I428Y/F, S492F, V495I/F/Y, P392Y, L621Q, D437H+D503Y, V674+L664F and L823V.

Disulfide bonds

A variant with improved stability (typically improved thermostability) as compared to the parent pullulanase may be obtained by introducing new interdomain and intradomain contacts, such as establishing inter- or intradomain disulfide bridges.

Accordingly, a further aspect of the present invention relates to a method for constructing a variant of a parent pullulanase, the method comprising:

a) identifying in the three-dimensional structure of the parent pullulanase two or more amino acid residues which, when substituted with cysteines, are capable of forming a disulfide bond;

b) substituting the amino acids identified in a) with cysteines;

c) optionally repeating steps a) and b) recursively;

d) optionally, making alterations each of which is an insertion, a deletion or a substitution of an amino acid residue at one or more positions other than b);

e) preparing the variant resulting from steps a) - d);

f) testing the stability of said variant; and

g) optionally repeating steps a) - f) recursively; and

h) selecting a variant having increased stability as compared to the parent pullulanase.

In a preferred embodiment of the invention the variant pullanase provided by the above method have increased thermostability as compared to the parent pullulanase. The thermostability of a given variant may be assessed as described in the above section entitled "Methods for determining stability, activity and specificity".

In order to determine, in the three-dimensional structure of the parent pullulanase, the amino acid residues which, when substituted with cysteines, are capable of forming a disulfide bond, residues with CB atoms less than 4Å from each other, and where the direction of the CA-CB from each residue is pointing towards the other residue are identified. Following the above-mentioned guidelines, the below amino acid residues were identified in the amino acid sequence of SEQ ID NO: 1, and it is contemplated that these residues are suitable for cystein replacement, thereby opening up the possibility of establishing one or more disulfide bridges in the variant pullulanase:

K758C+I914C, T916C+A765C, I897C+S819C, P525C+E499C, H286C+T148C.

Similar substitutions may be introduced in equivalent positions of other pullulanases. Variants of particular interest have a combination of one or more of the above with any of the other modifications disclosed herein.

For example, it is contemplated that the following residues, identified in the amino acid sequence of the pullulanase from *Bacillus deramificans* (SEQ ID NO: 3), are suitable for cystein replacement, thereby opening up the possibility of establishing one or more disulfide bridges in the variant pullulanase:

K756C/I912C, M914C/A763C, V895C/G817C, A523C/E497C, H284C/T144C.

Surface charge distribution

A variant with improved stability (typically improved thermostability) as compared to the parent pullulanase may be obtained by changing the surface charge distribution of the

pullulanase. For example, when the pH is lowered to about 5 or below histidine residues typically become positively charged and, consequently, unfavorable electrostatic interactions on the protein surface may occur. By engineering the surface charge of the pullulanase one may avoid such unfavorable electrostatic interactions which in turn leads to a higher stability of the pullulanase.

Therefore, a further aspect of the present invention relates to method for constructing a variant of a parent pullulanase, the method comprising:

a) identifying, on the surface of the parent pullulanase, at least one amino acid residue selected from the group consisting of Asp, Glu, Arg, Lys and His;

b) substituting, on the surface of the parent pullulanase, at least one amino acid residue selected from the group consisting of Asp, Glu, Arg, Lys and His with an uncharged amino acid residue;

c) optionally repeating steps a) and b) recursively;

d) optionally, making alterations each of which is an insertion, a deletion or a substitution of an amino acid residue at one or more positions other than b);

e) preparing the variant resulting from steps a) - d);

f) testing the stability of said variant; and

g) optionally repeating steps a) - f) recursively; and

h) selecting a variant having increased stability as compared to the parent pullulanase.

As will be understood by the skilled person it may also, in some cases, be advantageous to substitute an uncharged amino acid residue with an amino acid residue bearing a charge or, alternatively, it may in some cases be advantageous to substitute an amino acid residue bearing a charge with an amino acid residue bearing a charge of opposite sign. Thus, the above-mentioned method may easily be employed

by the skilled person also for these purposes. In the case of substituting an uncharged amino acid residue with an amino acid residue bearing a charge the above-mentioned method may be employed the only difference being steps a) and b) which will then read:

a) identifying, on the surface of the parent pullulanase, at least one uncharged amino acid residue;

b) substituting, on the surface of the parent pullulanase, at least one uncharged amino acid residue with a charged amino acid residue selected from the group consisting of Asp, Glu, Arg, Lys and His.

Also in the case of changing the sign of an amino acid residue present on the surface of the pullulanase the above method may be employed. Again, compared to the above method, the only difference being steps a) and b) which, in this case, read:

a) identifying, on the surface of the parent pullulanase, at least one charged amino acid residue selected from the group consisting of Asp, Glu, Arg, Lys and His;

b) substituting, on the surface of the parent pullulanase, at least one charged amino acid residue identified in step a) with an amino acid residue having an opposite charge.

Thus, Asp may be substituted with Arg, Lys or His; Glu may be substituted with Arg, Lys or His; Arg may be substituted with Asp or Glu; Lys may be substituted with Asp or Glu; and His may be substituted with Asp or Glu.

In a preferred embodiment of the invention the variant pullulanase provided by the above method(s) have increased thermostability as compared to the parent pullulanase. The thermostability of a given variant may be assessed as described in the above section entitled "Methods for determining stability, activity and specificity".

In order to determine the amino acid residues of a pullulanase, which are present on the surface of the enzyme, the surface accessible area are measured using the DSSP

program (Kabsch and Sander, *Biopolymers* (1983), **22**, 2577-2637). All residues having a surface accessibilty higher than 0 is regarded a surface residue.

The amino acid residues found on the surface of Promozyme® using the above method are as follows:

E526, Q544, E760, N338, N228, N181,

and it is contemplated that the following substitutions are of particular interest:

E526H, Q544E, E760Q, N338K/R, N228DE/, N181K/R.

Similar substitutions may be introduced in equivalent positions of other pullulanases. Variants of particular interest have a combination of one or more of the above with any of the other modifications disclosed herein.

For example, the variant of a pullulanase may also comprise one or more modifications, e.g. substitutions, corresponding to the following substitutions in the amino acid sequence set forth in SEQ ID NO: 3:

444, 530, 710 and 855.

In a more preferred embodiment, the variant of a pullulanse comprises one or more substitutions corresponding to the following substitutions in the amino acid sequence set forth in SEQ ID NO: 3:

D444R/K, K530Y/F/L, N710R and T855K.

Other modifications

Variants with improved stability, in particular variants with improved thermostability, can be obtained by improving existing or introducing new interdomain or intradomain contacts. Such improved stability can be achieved by the modifications listed below.

Thus, one preferred embodiment of the invention relates to a variant of a parent pullulanase which has an improved stability and one or more salt bridges as compared

to the parent pullulanase, wherein said variant comprises a modifications, e.g. a substitution, in a position corresponding to at least one of the following sets of positions in SEQ ID NO: 1:

301, 385, 298, 299, 385 and 299+385, in particular L301R, N385R, H298R, N299R, N385D and N299R+N385D.

Similar modifications, e.g. substitutions, may be introduced in equivalent positions of other pullulanases. Variants of particular interest have a combination of one or more of the above with any of the other modifications disclosed herein.

For example, it is contemplated that the following substitutions in the pullunanase having the amino acid sequence set forth in SEQ ID NO: 3 will enhance the stability of the enzyme: T891D, S892K, T891D+S892K and N400R.

In another preferred embodiment, the variant of the pullulanase comprises a substitution corresponding to one or more of the following substitutions with proline in the amino acid sequence set forth in SEQ ID NO: 1:

G293P, K151P, K122P, N315P, N374P, N793P, A446P, G672P, G668P, T556P

In a further interesting embodiment of the invention, the variant of the pullulanase comprises a substitution corresponding to one or more of the following substitutions with proline in the amino acid sequence set forth in SEQ ID NO: 3:

D562P, G794P, G292P, D148P, N119P, D314P, N373P, N792P, G671P, G667P and T554P.

Analogously, it may be preferred that one or more histidine residue(s) present in the parent pullulanase is (are) substituted with a non-histidine residues such as Y, V I, L, F, M, E, Q, N, or D. Accordingly, in another preferred embodiment, the variant of the parent pullulanase comprises a substitution of an amino acid residue corresponding to one or more of the following residues of the amino acid sequence set forth in SEQ ID NO: 3: H422Y/F/L, H483Y/F/L, H543Y/F/L/N and H613Y/F/L.

It may be preferred that one or more asparagine or glutamine residues present in the parent pullulanase is or are substituted with a residue lacking the amide group on the side chain. Preferably, such asparagines or glutamine residues are substituted with S, T, V, L and/or F amino acid residues. Accordingly, in another preferred embodiment, the variant of the parent pullulanase comprises a modification, e.g. a substitution, of an amino acid residue corresponding to one or more of the following residues of the amino acid sequence set forth in SEQ ID NO: 1:

Q543, Q339, N337, Q380, Q353, N384, N286, N298, N227, Q227, Q210, N180, Q259, N583, N790, N793, N505, N788, N736, N684, N689 or N681, preferably Q543S/T/V/L/F, Q339S/T/V/L/F, N337S/T/V/L/F, Q380S/T/V/L/F, Q353S/T/V/L/F, N384S/T/V/L/F, N286S/T/V/L/F, N298S/T/V/L/F, N227S/T/V/L/F, Q227S/T/V/L/F, Q210S/T/V/L/F, N180S/T/V/L/F, Q259S/T/V/L/F, N583S/T/V/L/F, N790S/T/V/L/F, N793S/T/V/L/F, N505S/T/V/L/F, N788S/T/V/L/F, N736S/T/V/L/F, N684S/T/V/L/F, N689S/T/V/L/F and N681S/T/V/L/F.

The corresponding residues found in the pullulanase from *Bacillus deramificans* (SEQ ID NO: 3) include:

N400, N446, N504, N717, N735 and N789, preferably N400S/T/V/L/F, N446S/T/V/L/F, N504S/T/V/L/F, N717S/T/V/L/F, N735S/T/V/L/F and N789S/T/V/L/F.

Moreover, it is contemplated that modifications, e.g. substitutions, in the region linking the N2 and the A domain, as well as other regions linking other domains, will confer additional stability, such as an increased thermostability, to the enzyme. Thus, in an interesting embodiment of the invention, the pullulanase variant comprises one or more modifications, e.g. substitutions, in the domain-linking regions (e.g. the region linking the N2 and A domains).

Examples of such modifications include one or more of the following substitutions in the pullulanase from *Bacillus deramificans* (SEQ ID NO: 3):

111, 112,

158-160 (i.e. 158, 159 and 160),

270-274 (i.e. 270, 271, 272, 273 and 274),

302-314 (i.e. 302, 303, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313 and 314) and

408-426 (i.e. 408, 409, 410, 411, 412, 413, 414, 415, 416, 417, 418, 419, 420, 421, 422, 423, 424, 425 and 426).

Examples of specific substitutions are: S111T/V/L, N112S/T/Q, S158Y/F/T, L159Y/K/R/A/S/T, G160A/S/T, D270E/S/T, L271V/I, V272I, T273N/D/E/Y/F, V274I, N302V/L/Y, N305V/L/Y, S306T/V, Q308K/R/A/S/T, Y309F, Y310E/D/Q/N/L/V/I, D314A/S/T, L409N, D408S/T, A410S/T, D413R/K/S/T, A415S/T, G416S/T/V, N418A/V/S/T, S419D/N/T, K421E/Q/S/T/V/A, H422D/L/Y/F, I423L/V/S/T/N/Q, T424S/A and K426A/S/T.

Other substitutions which are considered of particular importance in SEQ ID NO:3 include D437N and D440N.

Similar modifications, e.g. substitutions, may be introduced in equivalent positions of other pullulanases. Modifications of particular interest are any combination of one or more of the above with any of the other modifications disclosed herein.

Before actually constructing a pullulanase variant to achieve any of the above objectives, it may be convenient to evaluate whether or not the contemplated amino acid modification can be accommodated into pullulanase structure, e.g. in a model of the three-dimensional structure of the parent pullulanase.

Pullulanase variants with an altered substrate specificity

One aim of the present invention is to change the degradation characteristics of a pullulanase. Thus, as Promozyme® (and pullulanases in general) exhibits a low activity towards high molecular weight branched starchy material, such as glycogen and amylopectin, it may be desirable to change this cleavage pattern, e.g. so as to obtain a

higher activity against such substrates, in particular when the pullulanase is to be added during the liquefaction process.

An altered substrate specificity may be achieved by modifying the substrate binding area in a parent pullulanse.

Accordingly, the present invention also relates to a method for constructing a variant of a parent pullulanase, the method comprising:

a) identifying the substrate binding area in a model of the three-dimensional structure of the parent pullulanase;

b) modifying the substrate binding area by an amino acid substitution, deletion and/or insertion;

c) optionally repeating step b) recursively;

d) optionally, making alterations each of which is an insertion, a deletion or a substitution of an amino acid residue at one or more positions other than b),

e) preparing the variant resulting from steps a) - d);

f) testing the substrate specificity of the variant;

g) optionally repeating steps a) - f) recursively; and

h) selecting a variant having an altered substrate specificity as compared to the parent pullulanase.

The substrate binding area may easily be identified by homology to other family 13 members. The active site residues are identified by homology. The substrate-binding site is identified by the concave cavity containing the active site residues. A substrate model is docked into the cavity. A suitable substrate model is the substrate structure found in the pdb file 1BAG termed GLC. This model can be "docked" into the Promozyme X-ray structure or a modeled Pullulanase 3D structure by superimposing the active site residues in the two structures. In 1BAG one of the active site residues

has been mutated into an Gln instead of the native Glu. The active site residues to be superimposed are: D269, Q208 and D176 (1BAG) with D736, E651 and D622 (Promozyme®). The superposition can be made using the program INSIGHTII.

Without being limited to any theory, it is presently believed that binding between a substrate and an enzyme is supported by favorable interactions found within a sphere 10 Å from the substrate molecule, in particular within a sphere of 6 Å from the substrate molecule. Examples of such favorable bonds are hydrogen bonds, strong electrostatic interaction and/or hydrophobic interactions. The following residues of Promozyme® (SEQ ID NO: 1), are within a distance of 10 Å from the "docked" substrate and thus believed to be involved in interactions with said substrate:

437, 439, 487, 489, 490, 514, 679, 681, 684, 685, 731, 775, 786,

494-496 (i.e. 494, 495 and 496),

505-511 (i.e. 505, 506, 507, 508, 509, 510 and 511),

551-559 (i.e. 551, 552, 553, 554, 555, 556, 557, 558 and 559),

584-590 (i.e. 584, 585, 586, 587, 588, 589 and 590),

620-626 (i.e. 620, 621, 622, 623, 624, 625, 626),

650-658 (i.e. 659, 651, 652, 653, 654, 655, 656, 657 and 568),

665-668 (i.e. 666, 667 and 668),

690-693 (i.e. 690, 691, 692 and 693),

734-738 (i.e. 734, 735, 736, 737 and 738) and

789-795 (i.e. 789, 790, 791, 792, 793, 794 and 795).

The following residues of Promozyme® are within a distance of 6 Å from the substrate and thus believed to be involved in interactions with said substrate:

489, 551, 553, 555, 556, 620, 651, 691, 692, 791, 793, 794,

506-510 (i.e. 507, 508, 509 and 510),

586-588 (i.e. 586, 587 and 588),

622-624 (i.e. 622, 623 and 624),

653-656 (i.e. 653, 654, 655 and 656) and

735-737 (i.e. 735, 736 and 737),

In a preferred embodiment of the invention, the parent pullulanase is modified in such a way that the variant pulluanase exhibits an increased isoamylase activity compared to the parent pullulanase.

When used herein, the term "increased isoamylase activity" refers in general to the fact that the pullulanase variants according to the invention exhibits a higher activity towards high molecular weight branched starchy material, such as glycogen and amylopectin as compared to the parent pullulanase, cf. above.

In an interesting embodiment of the invention the pullulanase variant has an increased isoamylase activity as defined by an increase of at least 5%, preferably of at least 10%, more preferably of at least 15%, more preferably of at least 25%, most preferably of at least 50%, in particular of at least 75%, such as of at least 100% in the number of reducing ends formed in the "assay for isoamylase-like activity" described herein, using 50 mM sodium acetate, a pH of 4.5, 5.0 or 5.5, a temperature of 60°C and when incubated with a 10 w/v rabbit liver glycogen solution for a period of 10 min.

Similar modifications may be introduced in equivalent positions of other pullulanases. Substitutions of particular interest are any combination of one or both of the above with any of the other modifications disclosed herein.

For example, the following residues of the pullulanase from *Bacillus deramificans* (SEQ ID NO: 3) are within a distance of 10 Å from the "docked" substrate and thus believed to be involved in interactions with said substrate:

435, 437, 485, 487, 488, 512, 677, 679, 682, 683, 729, 773, 784,

492-494 (i.e. 492, 493 and 494),

503-509 (i.e. 503, 504, 505, 506, 507, 508 and 509),

549-557 (i.e. 549, 550, 551, 552, 553, 554, 555, 556 and 557),

582-588 (i.e. 582, 583, 584, 585, 586, 587 and 588),

618-624 (i.e. 618, 619, 620, 621, 622, 623 and 624),

648-656 (648, 649, 650, 651, 652, 653, 654, 655 and 656),

663-666 (i.e. 663, 664, 665 and 666),

688-691 (i.e. 688, 689, 690 and 691),
732-736 (732, 733, 734, 735 and 736) and

787-793 (i.e. 787, 788, 879, 790, 791, 792 and 793).

The following residues of the pullulanase from *Bacillus deramificans* (SEQ ID NO: 3) are within a distance of 6 Å from the substrate and thus believed to be involved in interactions with said substrate:

487, 549, 551, 553, 554, 618, 649, 689, 690, 789, 791, 792,

504-508 (i.e. 504, 505, 506, 507 and 508),

584-586 (i.e. 584, 585 and 586),

620-622 (i.e. 620, 621 and 622),

651-654 (i.e. 651, 652, 653 and 654) and

733-735 (i.e. 733, 734 and 735).

Examples of specific modifications in the above-mentioned regions of *Bacillus deramificans* are: L621I/V, D508M/N/L/T/V, T586I/L/V, T677W/F/Y, Y729F/I/L, D679G/A/V, S732V/T/L/I, N735G/L/V/I/S/T/A and Δ(688-691).

Pullulanase variants with altered pH dependent activity profile

The pH dependent activity profile can be changed by changing the pKa of residues within 15 Å, in particular by changing the pKa of residues within 10 Å, from the active site residues of the parent pullulanase. Changing the pKa of the active site residues is achieved, e.g., by changing the electrostatic interaction or hydrophobic interaction between functional groups of amino acid side chains of a given amino acid residue and its close surroundings. To obtain a higher activity at a higher pH, negatively charged residues are placed near a hydrogen donor acid, whereas positively charged residues placed near a nucleophilic acid will result in higher activity at low pH. Also, a decrease in the pKa can be obtained by reducing the accessibility of water or increasing hydrophobicity of the environment.

It is preferred that the variant in question exhibits a pH optimum which is at least about 0.5 pH units higher or lower, preferably at least about 1.0 pH units higher or lower, than the corresponding pH optimum of the parent pullulanase when tested on a suitable substrate (e.g. pullulan, amylopectin or glycogen).

Furthermore, it is particular preferred that the variant in question exhibits an increased activity in the pH range of from 4 to 5.5 as compared to the parent pullulanase when tested on a suitable substrate (e.g. pullulan, amylopectin or glycogen).

Thus, another aspect of the present invention relates to a method for constructing a variant of a parent pullulanase, the method comprising:

a) identifying an amino acid residue which is within 15 Å, in particular within 10 Å, from an active site residue of the parent pullulanase in the three-dimensional structure of said parent pullulanse, and which is involved in electrostatic or hydrophobic interactions with an active site residue;

b) substituting said amino acid residue with another amino acid residue which changes the electrostatic and/or hydrophobic surroundings of an active site residue, and which can be accommodated in the structure;

c) optionally repeating steps a) and b) recursively;

d) optionally, making alterations each of which is an insertion, a deletion or a substitution of an amino acid residue at one or more positions other than b);

e) preparing the variant resulting from steps a) - d);

f) testing the pH dependent activity of said variant; and

g) optionally repeating steps a) - f) recursively; and

h) selecting a variant having an altered pH dependent activity as compared to the parent amylase.

In general, an amino acid residue which is within 15 Å or 10Å, respectively, from an active site residue of the parent pullulanase may be identified by using the INSIGHTII program.

In a preferred embodiment, the variant of a parent pullulanase having an altered pH dependent activity profile as compared to the parent pullulanase comprises a

modification, e.g. a substitution, of an amino acid residue corresponding to one or more of the following residues of the amino acid sequence set forth in SEQ ID NO: 1 (all within 15Å from the active site residues D736, E651, D622):

430, 433, 518, 521, 565, 599, 600, 610, 611, 635, 636, 639, 717, 760, 763, 764, 767, 817,

435-443 (i.e. 435, 436, 437, 438, 439, 440, 441, 442, and 443),

486-496 (i.e. 486, 487, 488, 489, 490, 491, 492, 493, 494, 495 and 496),

505-515 (i.e. 505, 506, 507, 508, 509, 510, 511, 512, 513, 514 and 515),

548-560 (i.e. 548, 549, 550, 551, 552, 553, 554, 555, 556, 557, 558, 559 and 560),

573-575, (i.e. 573, 574 and 575),

583-595 (i.e. 583, 584, 585, 586, 587, 588, 589, 590, 591, 592, 593, 594 and 594),

602-604 (i.e. 602, 603 and 604),

606-608 (i.e. 606-607 and 608),

616-633 (i.e. 616, 617, 618, 619, 620, 621, 622, 623, 624, 625, 626, 627, 628, 629, 630, 631, 632, and 633),

646-672 (i.e. 646, 647, 648, 649, 650, 651, 652, 653, 654, 655, 656, 657, 658, 659, 660, 661, 662, 663, 664, 665, 666, 667, 668, 669, 670, 671 and 672),

674-696 (i.e. 674, 675, 676, 677, 678, 679, 680, 681, 682, 683, 684, 685, 686, 687, 688, 689, 690, 691, 692, 693, 694, 695 and 696),

720-722 (i.e. 720, 721 and 722),

725-747 (i.e. 725, 726, 727, 728, 729, 730, 731, 732, 733, 734, 735, 736, 737, 738, 739, 740, 741, 742, 743, 744, 745, 746 and 747),

773-781 (i.e. 773, 774, 775, 776, 777, 778, 779, 780 and 781),

783-797 (i.e. 783, 784, 785, 786, 787, 788, 789, 790, 791, 792, 793, 794, 795, 796 and 797) and

799-802 (i.e. 799, 800, 801 and 802).

Within 10Å from the active site residues D736, E651, D622:

437, 442, 492, 514, 575, 594, 603, 632, 635, 684, 688, 691, 692, 721, 727, 729, 742, 743, 775, 777, 778, 780, 784, 786, 800,

487-490 (i.e. 487, 488, 489 and 490),

507-511 (i.e. 507, 508, 509, 510 and 511),

550-557 (i.e. 550, 551, 552, 553, 554, 555, 556 and 556),

585-588 (i.e. 585, 586, 587 and 588),

590-592 (i.e. 590, 591 and 592),

619-628 (i.e. 619, 620, 621, 622, 623, 624, 625, 626, 627 and 628),

648-655 (i.e. 648, 649, 650, 651, 652, 653, 654 and 655),

665-671 (i.e. 665, 666, 667, 668, 669, 670 and 671),

676-681 (i.e. 676, 677, 678, 679, 680 and 681),

731-740 (i.e. 731, 732, 733, 734, 735, 736, 737, 738, 739 and 740) and

788-793 (i.e. 788, 789, 790, 791, 792 and 793).

Similar modifications may be introduced in equivalent positions of other pullulanases. Variants of particular interest have a combination of one or more of the above with any of the other modifications disclosed herein.

Thus, in another preferred embodiment, the variant of a parent pullulanase having an altered pH dependent activity profile as compared to the parent pullulanase comprises a modification, e.g. a substitution, of an amino acid residue corresponding to one or more of the following residues of the amino acid sequence set forth in SEQ ID NO: 3 (all within 15 Å from the active site residues D734, E649 and D620):

428, 431, 516, 519, 563, 597, 598, 608, 609, 633, 634, 637, 715, 758, 761, 762, 765, 815,

433-441 (i.e. 433, 434, 435, 436, 437, 438, 439, 440 and 441),

484-494 (i.e. 484, 485, 486, 487, 488, 489, 490, 491, 492, 493 and 494),

503-513 (i.e. 503, 504, 505, 506, 507, 508, 509, 510, 511, 512 and 513),

546-558 (546, 547, 548, 549, 550, 551, 552, 553, 554, 555, 556, 557 and 558),

571-573 (i.e. 571, 572 and 573),

581-593 (i.e. 581, 582, 583, 584, 585, 586, 587, 588, 589, 590, 591, 592 and 593),

600-602 (i.e. 600, 601 and 602),

604-606 (i.e. 604, 605 and 606),

614-631 (i.e. 614, 615, 616, 617, 618, 619, 620, 621, 622, 623, 624, 625, 626, 627, 628, 629, 630 and 631),

644-670 (i.e. 644, 645, 646, 647, 648, 649, 650, 651, 652, 653, 654, 655, 656, 657, 658, 659, 660, 661, 662, 663, 664, 665, 666, 667, 668, 669 and 670),

672-694 (i.e. 672, 673, 674, 675, 676, 677, 678, 679, 680, 681, 682, 683, 684, 685, 686, 687, 688, 689, 690,691, 692, 693 and 694),

718-720 (i.e. 718, 719 and 720),

723-745 (i.e. 723, 734, 725, 726, 727, 728, 729, 730, 731, 732, 733, 734, 735, 736, 737, 738, 739, 740, 741, 742, 743, 744 and 745),

771-779 (i.e. 771, 772, 773, 774, 775, 776, 777, 778 and 779),

781-795 (i.e. 781, 782, 783, 784, 785, 786, 787, 788, 789, 790, 791, 792, 793, 794 and 795) and

797-800 (i.e. 797, 798, 799 and 800).

Within 10 Å from the active site residues D734, E649 and D620:

435, 440, 490, 512, 573, 601, 605, 630, 669, 682, 686, 689, 690, 719, 725, 727, 740, 741, 773, 775, 776, 778, 782, 784, 798,

485-488 (i.e. 485, 486, 487 and 488),

505-509 (i.e. 505, 506, 507, 508 and 509),

548-555 (i.e. 548, 549, 550, 551, 552, 553, 554 and 555),

583-586 (i.e. 583, 584, 585 and 586),

588-590 (i.e. 588, 589 and 590),

617-626 (i.e. 616, 617, 618, 619, 620, 621, 622, 623, 624, 625 and 626),

646-653 (i.e. 646, 647, 648, 649, 650, 651, 652 and 653),

663-667 (i.e. 663, 664, 665, 666 and 667),

674-679 (i.e. 674, 675, 676, 677, 678 and 679),

729-738 (i.e. 729, 730, 731, 732, 733, 734, 735, 736, 737 and 738) and

786-791 (i.e. 786, 787, 788, 789, 790 and 791).

Specific examples of substitutions in the above-mentioned positions include D437L/I/V/F, D440L/I/V/F, M486K, M487K, D503L/I/V/F, D508N/L/T/V, T586V/I, M630H and D437L/I/V/F+D440L/I/V/F+D503L/I/V/F.

Nomenclature for amino acid modifications

The nomenclature used herein for defining modifications is essentially as described in WO 92/05249. Thus, G406A indicates a substitution of the amino acid G (Gly) in position 406 with the amino acid A (Ala). G406 indicates a substitution of the amino acid G (Gly) with any other amino acid. P394F/W/I/L indicates a substitution of P394 with F, W, I or L. Δ(688-691) indicates a deletion of amino acids in positions 688-691. 412-A-413 indicates an insertion of A between amino acids 412 and 413.

When used herein, the term "modification" (of a particular amino acid residue) is intended to cover substitution and deletion (of the particular amino acid residue) as well as insertion of one or more amino acid residues after the particular amino acid residue.

Polypeptide sequence homology

For purposes of the present invention, the degree of homology may be suitably determined according to the method described in S.B. Needleman and C.D. Wunsch, *Journal of Molecular Biology*, **48**, 443-45, with the following settings for polypeptide sequence comparison: GAP creation penalty of 3.0 and GAP extension penalty of 0.1. The determination may be done by means of a computer program known such as GAP provided in the UWGCG program package (Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711).

Hybridization

Suitable experimental conditions for determining hybridization between a nucleotide probe and a homologous DNA or RNA sequence involves presoaking of the filter containing the DNA fragments or RNA to hybridize in 5x SSC (sodium chloride/sodium citrate, Sambrook, et al. Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor, 1989) for 10 min, and prehybridization of the filter in a solution of 5x SSC, 5x Denhardt's solution (Sambrook, et al., 1989), 0.5% SDS and 100 µg/ml of denatured sonicated salmon sperm DNA (Sambrook, et al., 1989), followed by hybridization in the same solution containing a random-primed (A. P. Feinberg B. and Vogelstein, *Anal. Biochem.* **132**, 6-13 (1983)), ^{32}P-dCTP-labeled (specific activity > 1 x 10^9 cpm/µg) probe for 12 hours at ca. 45°C. The filter is then washed twice for 30 minutes in 2x SSC, 0.5% SDS at least 55°C (low stringency), preferably at least 60°C (medium stringency), more preferably at least 65°C (medium/high stringency), more preferably at least 70°C (high stringency), even more preferably at least 75°C (very high stringency).

Molecules which hybridize to the oligonucleotide probe under these conditions are detected by exposure to x-ray film.

Methods of preparing pullulanase variants according to the invention

Cloning a DNA sequence encoding a pullulanase

The DNA sequence encoding a parent pullulanase may be isolated from any cell or microorganism producing the pullulanase in question, using various methods well known in the art.

First, a genomic DNA and/or cDNA library should be constructed using chromosomal DNA or messenger RNA from the organism that produces the pullulanase to be studied. Then, if the amino acid sequence of the pullulanase is known, homologous, labelled oligonucleotide probes may be synthesised and used to identify pullulanase-encoding clones from a genomic library prepared from the organism in question. Alternatively, a labelled oligonucleotide probe containing sequences homologous to a known pullulanase gene could be used as a probe to identify pullulanase-encoding clones, using hybridization and washing conditions of lower stringency.

Alternatively, the DNA sequence encoding the enzyme may be prepared synthetically by established standard methods, e.g. the phosphoroamidite method described by S.L. Beaucage and M.H. Caruthers, *Tetrahedron Letters*, **22**, 1859-1869 (1981) or the method described by Matthes et al. *The EMBO*, **3**, 801-805 (1984). In the phosphoroamidite method, oligonucleotides are synthesized, e.g. in an automatic DNA synthesizer, purified, annealed, ligated and cloned in appropriate vectors.

Finally, the DNA sequence may be of mixed genomic and synthetic origin, mixed synthetic and cDNA origin or mixed genomic and cDNA origin, prepared by ligating fragments of synthetic, genomic or cDNA origin, wherein the fragments correspond to various parts of the entire DNA sequence, in accordance with techniques well known in the art. The DNA sequence may also be prepared by polymerase chain reaction (PCR) using specific primers, for instance as described in US 4,683,202 or R.K. Saiki et al. *Science*, **239**, 487-491(1988).

Site-directed Mutagenesis

Once a pullulanase-encoding DNA sequence has been isolated, and desirable sites for modification identified, modifications may be introduced using synthetic oligonucleotides. These oligonucleotides contain nucleotide sequences flanking the desired modification sites; mutant nucleotides are inserted during oligonucleotide synthesis. In a specific method, a single-stranded gap of DNA, bridging the pullulanase-encoding sequence, is created in a vector carrying the pullulanase gene. Then the synthetic nucleotide, bearing the desired modification, is annealed to a homologous portion of the single-stranded DNA. The remaining gap is then filled in with DNA polymerase I (Klenow fragment) and the construct is ligated using T4 ligase. A specific example of this method is described in Morinaga et al. *Biotechnology* **2**, 639-646 (1984). US 4,760,025 discloses the introduction of oligonucleotides encoding multiple modifications by performing minor alterations of the cassette. However, an even greater variety of modifications can be introduced at any one time by the Morinaga method because a multitude of oligonucleotides, of various lengths, can be introduced.

Another method of introducing modifications into a pullulanase-encoding DNA sequences is described in Nelson and Long *Analytical Biochemistry*, **180**, 147-151 (1989). It involves a 3-step generation of a PCR fragment containing the desired modification introduced by using a chemically synthesized DNA strand as one of the primers in the PCR reactions. From the PCR-generated fragment, a DNA fragment carrying the modification may be isolated by cleavage with restriction endonucleases and reinserted into an expression plasmid.

Random Mutagenesis

Random mutagenesis is suitably performed either as localized or region-specific random mutagenesis in at least three parts of the gene translating to the amino acid sequence shown in question, or within the whole gene.

The random mutagenesis of a DNA sequence encoding a parent pullulanase may be conveniently performed by use of any method known in the art.

In relation to the above, a further aspect of the present invention relates to a method for generating a variant of a parent pullulanase, wherein the variant exhibits an altered property, such as increased thermostability, increased stability at low pH and at low calcium concentration, relative to the parent pullulanase, the method comprising:

(a) subjecting a DNA sequence encoding the parent pullulanase to random mutagenesis,

(b) expressing the mutated DNA sequence obtained in step (a) in a host cell, and

(c) screening for host cells expressing a pullulanase variant which has an altered property relative to the parent pullulanase.

Step (a) of the above method of the invention is preferably performed using doped primers.

For instance, the random mutagenesis may be performed by use of a suitable physical or chemical mutagenizing agent, by use of a suitable oligonucleotide, or by subjecting

the DNA sequence to PCR generated mutagenesis. Furthermore, the random mutagenesis may be performed by use of any combination of these mutagenizing agents. The mutagenizing agent may, e.g., be one which induces transitions, transversions, inversions, scrambling, deletions, and/or insertions.

Examples of a physical or chemical mutagenizing agent suitable for the present purpose include ultraviolet (UV) irradiation, hydroxylamine, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), O-methyl hydroxylamine, nitrous acid, ethyl methane sulphonate (EMS), sodium bisulphite, formic acid, and nucleotide analogues. When such agents are used, the mutagenesis is typically performed by incubating the DNA sequence encoding the parent enzyme to be mutagenized in the presence of the mutagenizing agent of choice under suitable conditions for the mutagenesis to take place, and selecting for mutated DNA having the desired properties.

When the mutagenesis is performed by the use of an oligonucleotide, the oligonucleotide may be doped or spiked with the three non-parent nucleotides during the synthesis of the oligonucleotide at the positions which are to be changed. The doping or spiking may be done so that codons for unwanted amino acids are avoided. The doped or spiked oligonucleotide can be incorporated into the DNA encoding the pullulaase enzyme by any published technique, using e.g. PCR, LCR or any DNA polymerase and ligase as deemed appropriate.

Preferably, the doping is carried out using "constant random doping", in which the percentage of wild-type and modification in each position is predefined. Furthermore, the doping may be directed toward a preference for the introduction of certain nucleotides, and thereby a preference for the introduction of one or more specific amino acid residues. The doping may be made, e.g., so as to allow for the introduction of 90% wild type and 10% modifications in each position. An additional consideration in the choice of a doping scheme is based on genetic as well as protein-structural constraints. The doping scheme may be made by using the DOPE program which, *inter alia*, ensures that introduction of stop codons is avoided (L.J. Jensen et al. *Nucleic Acid Research*, **26**, 697-702 (1998).

When PCR-generated mutagenesis is used, either a chemically treated or non-treated gene encoding a parent pullulanase enzyme is subjected to PCR under conditions that increase the misincorporation of nucleotides (Deshler 1992; Leung et al., *Technique*, **1**, 1989, pp. 11-15).

A mutator strain of *E. coli* (Fowler et al., *Molec. Gen. Genet.*, **133**, 1974, 179-191), *S. cereviseae* or any other microbial organism may be used for the random mutagenesis of the DNA encoding the pullulanase by, e.g., transforming a plasmid containing the parent enzyme into the mutator strain, growing the mutator strain with the plasmid and isolating the mutated plasmid from the mutator strain. The mutated plasmid may be subsequently transformed into the expression organism.

The DNA sequence to be mutagenized may conveniently be present in a genomic or cDNA library prepared from an organism expressing the parent pullulanase. Alternatively, the DNA sequence may be present on a suitable vector such as a plasmid or a bacteriophage, which as such may be incubated with or otherwise exposed to the mutagenising agent. The DNA to be mutagenized may also be present in a host cell either by being integrated in the genome of said cell or by being present on a vector harbored in the cell. Finally, the DNA to be mutagenized may be in isolated form. It will be understood that the DNA sequence to be subjected to random mutagenesis is preferably a cDNA or a genomic DNA sequence.

In some cases it may be convenient to amplify the mutated DNA sequence prior to performing the expression step b) or the screening step c). Such amplification may be performed in accordance with methods known in the art, the presently preferred method being PCR-generated amplification using oligonucleotide primers prepared on the basis of the DNA or amino acid sequence of the parent enzyme.

Subsequent to the incubation with or exposure to the mutagenising agent, the mutated DNA is expressed by culturing a suitable host cell carrying the DNA sequence under conditions allowing expression to take place. The host cell used for this purpose may be one which has been transformed with the mutated DNA sequence, optionally present on a vector, or one which was carried the DNA sequence encoding the parent enzyme

during the mutagenesis treatment. Examples of suitable host cells are the following: gram positive bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus megaterium*, *Bacillus thuringiensis*, *Streptomyces lividans* or *Streptomyces murinus*; and gram negative bacteria such as *E. coli*.

The mutated DNA sequence may further comprise a DNA sequence encoding functions permitting expression of the mutated DNA sequence.

Localized random mutagenesis

The random mutagenesis may be advantageously localized to a part of the parent pullulanase in question. This may, e.g., be advantageous when certain regions of the enzyme have been identified to be of particular importance for a given property of the enzyme, and when modified are expected to result in a variant having improved properties. Such regions may normally be identified when the tertiary structure of the parent enzyme has been elucidated and related to the function of the enzyme.

The localized, or region-specific, random mutagenesis is conveniently performed by use of PCR generated mutagenesis techniques as described above or any other suitable technique known in the art. Alternatively, the DNA sequence encoding the part of the DNA sequence to be modified may be isolated, e.g., by insertion into a suitable vector, and said part may be subsequently subjected to mutagenesis by use of any of the mutagenesis methods discussed above.

General method for random mutagenesis by use of the DOPE program

The random mutagenesis may be carried out by the following steps:

1. Select regions of interest for modification in the parent enzyme

2. Decide on mutation sites and non-mutated sites in the selected region

3. Decide on which kind of mutations should be carried out, e.g. with respect to the desired stability and/or performance of the variant to be constructed

4. Select structurally reasonable mutations

5. Adjust the residues selected by step 3 with regard to step 4.

6. Analyze by use of a suitable dope algorithm the nucleotide distribution.

7. If necessary, adjust the wanted residues to genetic code realism, e.g. taking into account constraints resulting from the genetic code, e.g. in order to avoid introduction of stop codons; the skilled person will be aware that some codon combinations cannot be used in practice and will need to be adapted

8. Make primers

9. Perform random mutagenesis by use of the primers

10. Select resulting pullulanase variants by screening for the desired improved properties.

Suitable dope algorithms for use in step 6 are well known in the art. One such algorithm is described by Tomandl, D. et al., 1997, Journal of Computer-Aided Molecular Design 11:29-38. Another algorithm is DOPE (Jensen, LJ, Andersen, KV, Svendsen, A, and Kretzschmar, T (1998) Nucleic Acids Research 26:697-702).

Expression of pullulanase variants

The construction of the variant of interest is accomplished by cultivating a microorganism comprising a DNA sequence encoding the variant under conditions which are conducive for producing the variant, and optionally subsequently recovering the variant from the resulting culture broth. This is described in detail further below.

According to the invention, a DNA sequence encoding the variant produced by methods described above, or by any alternative methods known in the art, can be expressed, in the form of a protein or polypeptide, using an expression vector which typically includes

control sequences encoding a promoter, operator, ribosome binding site, translation initiation signal, and, optionally, a repressor gene or various activator genes.

The recombinant expression vector carrying the DNA sequence encoding an pullulanase variant of the invention may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid, a bacteriophage or an extrachromosomal element, minichromosome or an artificial chromosome. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the DNA sequence should be operably connected to a suitable promoter sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA sequence encoding a pullulanase variant of the invention, especially in a bacterial host, are the promoter of the *lac* operon of *E.coli*, the *Streptomyces coelicolor* agarase gene *dag*A promoters, the promoters of the *Bacillus licheniformis* α-amylase gene (*amyL*), the promoters of the *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), the promoters of the *Bacillus amyloliquefaciens* α-amylase (*amyQ*), the promoters of the *Bacillus subtilis* xylA and xylB genes, etc. For transcription in a fungal host, examples of useful promoters are those derived from the gene encoding *A. oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *A. niger* neutral α-amylase, *A. niger* acid stable α-amylase, *A. niger* glucoamylase, *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase or *A. nidulans* acetamidase.

The expression vector of the invention may also comprise a suitable transcription terminator and, in eukaryotes, polyadenylation sequences operably connected to the

DNA sequence encoding the pullulanase variant of the invention. Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter.

The vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. Examples of such sequences are the origins of replication of plasmids pUC19, pACYC177, pUB110, pE194, pAMB1 and pIJ702.

The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the *dal* genes from *B. subtilis* or *B. licheniformis*, or one which confers antibiotic resistance such as ampicillin, kanamycin, chloramphenicol or tetracycline resistance. Furthermore, the vector may comprise *Aspergillus* selection markers such as amdS, argB, niaD and sC, a marker giving rise to hygromycin resistance, or the selection may be accomplished by co-transformation, e.g. as described in WO 91/17243.

While intracellular expression may be advantageous in some respects, e.g. when using certain bacteria as host cells, it is generally preferred that the expression is extracellular. In general, the *Bacillus* α-amylases mentioned herein comprise a preregion permitting secretion of the expressed protease into the culture medium. If desirable, this preregion may be replaced by a different preregion or signal sequence, conveniently accomplished by substitution of the DNA sequences encoding the respective preregions.

The procedures used to ligate the DNA construct of the invention encoding the pullulanase variant, the promoter, terminator and other elements, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al. Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor, 1989).

The cell of the invention, either comprising a DNA construct or an expression vector of the invention as defined above, is advantageously used as a host cell in the recombinant production of a pullulanase variant of the invention. The cell may be

transformed with the DNA construct of the invention encoding the variant, conveniently by integrating the DNA construct (in one or more copies) in the host chromosome. This integration is generally considered to be an advantage as the DNA sequence is more likely to be stably maintained in the cell. Integration of the DNA constructs into the host chromosome may be performed according to conventional methods, e.g. by homologous or heterologous recombination. Alternatively, the cell may be transformed with an expression vector as described above in connection with the different types of host cells.

The cell of the invention may be a cell of a higher organism such as a mammal or an insect, but is preferably a microbial cell, e.g. a bacterial or a fungal (including yeast) cell.

Examples of suitable bacteria are gram positive bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus megaterium*, *Bacillus thuringiensis*, or *Streptomyces lividans* or *Streptomyces murinus*, or gram negative bacteria such as *E.coli*. The transformation of the bacteria may, for instance, be effected by protoplast transformation or by using competent cells in a manner known *per se*.

The yeast organism may favourably be selected from a species of *Saccharomyces* or *Schizosaccharomyces*, e.g. *Saccharomyces cerevisiae*. The filamentous fungus may advantageously belong to a species of *Aspergillus*, e.g. *Aspergillus oryzae* or *Aspergillus niger*. Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known *per se*. A suitable procedure for transformation of *Aspergillus* host cells is described in EP 238 023.

In a yet further aspect, the present invention relates to a method for producing a pullulanase variant of the invention, the method comprising: cultivating a host cell as described above under conditions conducive to the production of the variant and recovering the variant from the cells and/or culture medium.

The medium used to cultivate the cells may be any conventional medium suitable for growing the host cell in question and obtaining expression of the pullulanase variant of the invention. Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g. as described in catalogues of the American Type Culture Collection).

The pullulanase variant secreted from the host cells may conveniently be recovered from the culture medium by well-known procedures, including separating the cells from the medium by centrifugation or filtration, and precipitating proteinaceous components of the medium by means of a salt such as ammonium sulfate, followed by the use of chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

Testing of pullulanase

Pullulanase variants produced by any of the methods described above may be tested, either prior to or after purification, for pullulanase activity in a screening assay which measures the ability of the variant to degrade pullulan or, in case it is desired to screen for an increased isoamylases activity, the ability of the variant to degrade amylopectin. The screening in step 10 in the above-mentioned random mutagenesis method of the invention may be conveniently performed by use of a filter assay based on the following procedure: A microorganism capable of expressing the mutated pullulanase of interest is incubated on a suitable medium and under suitable conditions for secretion of the enzyme, the medium being covered with two filters comprising a protein-binding filter placed under a second filter exhibiting a low protein binding capability. The microorganism is grown on the second, top filter. Subsequent to the incubation, the bottom protein-binding filter comprising enzymes secreted from the microorganism is separated from the second filter comprising the microorganism. The protein-binding filter is then subjected to screening for the desired enzymatic activity, and the corresponding microbial colonies present on the second filter are identified. The first filter used for binding the enzymatic activity may be any protein-binding filter, e.g., nylon or nitrocellulose. The second filter carrying the colonies of the expression organism may

be any filter that has no or low affinity for binding proteins, e.g., cellulose acetate or Durapore™.

Screening consists of treating the first filter to which the secreted protein is bound with a substrate that allows detection of the activity. The enzymatic activity may be detected by a dye, fluorescence, precipitation, pH indicator, IR-absorbance or any other known technique for detection of enzymatic activity. The detecting compound may be immobilized by any immobilizing agent e.g. agarose, agar, gelatine, polyacrylamide, starch, filter paper, cloth; or any combination of immobilizing agents. For example, isoamylase activity can be detected by Cibacron Red labelled amylopectin, which is immobilized in agarose. isoamylase activity on this substrate produces zones on the plate with reduced red color intensity (clearing zones).

To screen for variants with increased stability, the filter with bound pullulanase variants can be pretreated prior to the detection step described above to inactivate variants that do not have improved stability relative to the parent pullulanase. This inactivation step may consist of, but is not limited to, incubation at elevated temperatures in the presence of a buffered solution at any pH from pH 2 to 12, and/or in a buffer containing another compound known or thought to contribute to altered stability e.g., surfactants, EDTA, EGTA, wheat flour components, or any other relevant additives. Filters so treated for a specified time are then rinsed briefly in deionized water and placed on plates for activity detection as described above. The conditions are chosen such that stabilized variants show increased enzymatic activity relative to the parent after incubation on the detection media.

To screen for variants with altered thermostability, filters with bound variants are incubated in buffer at a given pH (e.g., in the range from pH 2-12) at an elevated temperature (e.g., in the range from 50°-110°C) for a time period (e.g., from 1-20 minutes) to inactivate nearly all of the parent pullulanase, rinsed in water, then placed directly on a detection plate containing immobilized Cibacron Blue labeled pullulan and incubated until activity is detectable. As will be understood, thermostability and increased isoamylase activity may be tested simultaneously by using a detection plate

containing immobilized Cibacron Red labeled amylopectin and incubate until activity is detectable. Moreover, pH dependent stability can be screened for by adjusting the pH of the buffer in the above inactivation step such that the parent pullulanase is inactivated, thereby allowing detection of only those variants with increased stability at the pH in question. To screen for variants with increased calcium-dependent stability, calcium chelators, such as ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), is added to the inactivation buffer at a concentration such that the parent pullulanase is inactivated under conditions further defined, such as buffer pH, temperature or a specified length of incubation.

The variants of the invention may be suitably tested by assaying the pullulan- or amylopectin-degrading activity of the variant, for instance by growing host cells transformed with a DNA sequence encoding a variant on a starch-containing agarose plate and identifying pullulan- and/or amylopectin-degrading host cells as described above. Further testing in regard to altered properties, including specific activity, substrate specificity, cleavage pattern, thermoactivation, thermostability, pH dependent activity or optimum, pH dependent stability, temperature dependent activity or optimum, transglycosylation activity, stability, and any other parameter of interest, may be performed on purified variants in accordance with methods known in the art as described below.

The invention is further illustrated with reference to the following examples which are not intended to be in any way limiting to the scope of the invention as claimed.

EXAMPLES

Determination of pullulanase activity

Endo-pullulanase activity in NPUN is measured relative to a Novo Nordisk pullulanase standard. One pullulanase unit (NPUN) is defined as the amount of enzyme which releases 1 mmol glucose per minute under the standard conditions (0.7% red pullulan, pH 5, 40°C, 20 minutes). The activity is measured in NPUN/ml using red pullulan.

1 ml diluted sample or standard is incubated at 40°C for 2 minutes. 0.5 ml 2% red pullulan, 0.5 M KCl, 50 mM citric acid, pH 5 are added and mixed. The tubes are incubated at 40°C for 20 minutes and stopped by adding 2.5 ml 80% ethanol. The tubes are left standing at room temperature for 10-60 minutes followed by centrifugation 10 minutes at 4000 rpm. OD of the supernatants is then measured at 510 nm and the activity calculated using a standard curve.

Expression of pullulanase from *Bacillus deramificans*

The pullulanase from *Bacillus deramificans* (SEQ ID NO: 3) is expressed in *B. subtilis* from a plasmid denoted pCA36. This plasmid contains the complete gene encoding the pullulanase, the expression of which is directed by the promoter from *Bacillus amyloliquefaciens* α-amylase. Further, the plasmid contains the origin of replication, *oriT*, from plasmid pUB110 and the *cat* gene from plasmid pC194 conferring resistance towards chloramphenicol. PCA36 is shown in Fig. 1.

Example 1: Construction of *Bacillus deramificans D620A variant*

Gene specific primer 132011 and mutagenic primer 132012 are used to amplify by PCR an approximately 410 bp DNA fragment from the pCA36 plasmid.

The 410 bp fragment is purified from an agarose gel and used as a Mega-primer together with primer 136054 in a second PCR carried out on the same template.

The resulting approximately 1110 bp fragment is digested with restriction enzymes BsiW I and Mlu I and the resulting approximately 330 bp DNA fragment is purified and ligated with the pCA36 plasmid digested with the same enzymes. Competent *Bacillus subtilis* SHA273 (amylase and protease low) cells are transformed with the ligation and chlorampenicol resistant transformants are checked by colony PCR.

The mutagenesis primer 132012 introduced the D620A substitution (written in bold in the primer seq.) and introduced simultaneously a Bgl I restriction site (underlined in the primer seq.), which facilitates easy pinpointing of mutants.

Finally, DNA sequencing was carried out to verify the presence of the correct mutations on the plasmid.

Primer 132011:

5' CGCTTCGGAATCATTAGGATTGC 3'

Primer 132012:

5' GCTTCCGTTTT**GCC**TTAATGGCGCTGC 3'

Primer 136054:

5' GGCCAAGGCTCTACCCGAACGGC 3'

Example 2: Construction of *Bacillus deramificans E649A variant*

This variant constructed as described in Example 1, except that mutagenic primer 132013 is used. The mutagenesis primer 132013 introduced the E649A substitution (written in bold in the primer seq.) and a Nar I restriction site(underlined in the primer seq.), which facilitates easy pinpointing of mutants.

Primer 132013:

5' GCACTTTACGGG**GCG**CCATGGACGGG 3'